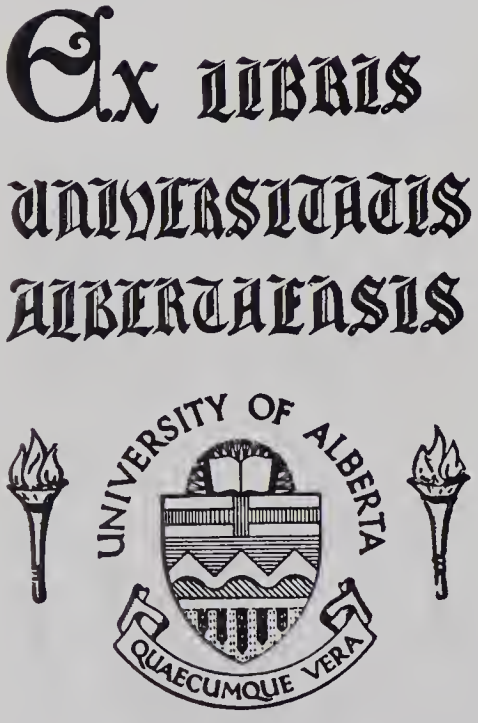

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Toxicity of Tri-o-cresyl Phosphate in Cats

by

Harpal Singh Buttar

A Thesis

Submitted to the Faculty of Graduate Studies in

Partial Fulfilment of the Requirements for the

Degree of Master of Science

Department of Pharmacology

Edmonton, Alberta

June, 1966

University of Alberta

Faculty of Graduate Studies

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Toxicity of Tri-o-cresyl phosphate in Cats" submitted by Harpal Singh Buttar in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Eto, Casida and Eto (1962) presented evidence that tri-o-cresyl phosphate (TOCP) is metabolized in rats to yield 2-(o-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one. They designated this substance, metabolite 1 or M-1 and the synthetic product SM-1. The synthetic product (SM-1) has been shown to cause ataxia, demyelination and nerve fiber degeneration in hens (Baron et al., 1962) and cats (Taylor, 1965 b).

This work was undertaken to determine whether the cat was able to metabolize tri-o-cresyl phosphate (TOCP) to a substance similar to 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one. Cats were treated with TOCP or TOCP-H^3 both by the intramuscular and intraperitoneal routes. A 5 per cent emulsion of TOCP-H^3 in 10 per cent tween 80 gave satisfactory results following intraperitoneal administration of the poison. Atropine sulphate (2.5 mg/kg) was injected subcutaneously together with the TOCP to minimize cholinergic symptoms. At varying times after the administration of TOCP or TOCP-H^3, the small intestines were removed and extracted with acetone. The extracted material was purified by adsorption and partition column chromatography. Fractions thus collected were subjected to ultraviolet spectrophotometry, liquid scintillation counting, and thin layer chromatography to detect the presence of the metabolite under investigation.

It was possible to obtain only a partial separation of TOCP from SM-1 by adsorption column chromatography. In this regard thin layer chromatography on silica gel G proved quite effective in resolving TOCP from SM-1 or M-1. Exposure to ultraviolet light and reaction of SM-1 or M-1 with 0.05 N $KMnO_4$ and 0.03 N H_2SO_4 were used to differentiate the TOCP spots from its metabolite.

When SM-1 was added to the sliced cat gut, marked losses of SM-1 were obtained during the process of extraction and purification.

ACKNOWLEDGEMENTS

I owe my sincere thanks and appreciation to Dr. Jack Dean Taylor, Ph.D.; for guidance, inspiration and encouragement throughout this investigation. His assistance in the preparation and organization of this dissertation is also gratefully acknowledged. Thanks are also due to Mrs June M. Vos for efficiently typing the final copy and to Mr. J. Mares for preparing the graphs. I am also grateful to the Vivarium staff for looking after the animals.

Finally, I would like to express my gratitude to the Medical Research Council of Canada and the University of Alberta for financial support.

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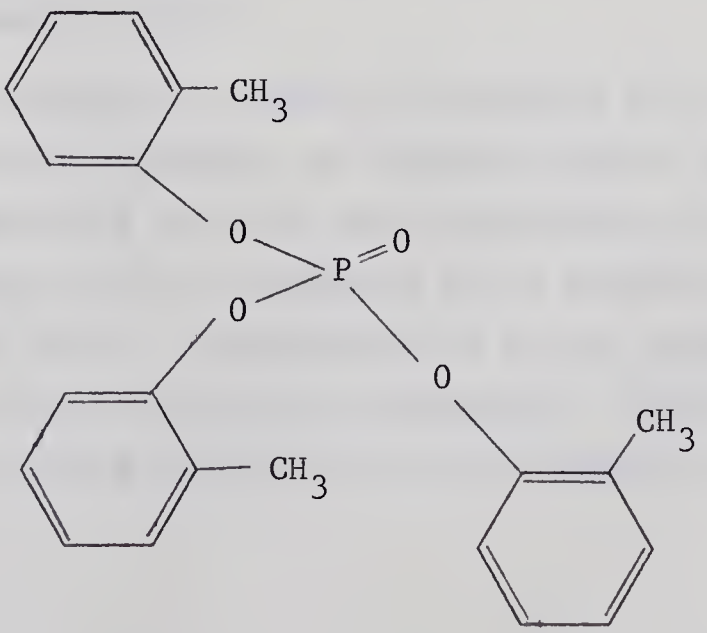
INTRODUCTION

"In the present complex civilization poisons lurk in preparations on every hand and the toxic properties of many of them are not suspected". (Kidd and Langworthy, 1933).

1. Triorthocresyl phosphate is a colourless, odourless, tasteless, viscid fluid having a low vapour pressure. Only the ortho isomer possesses the ability to cause demyelination of nerve fibres; the meta and para isomers are relatively non-toxic (Kidd and Langworthy, 1933). Tri-cresyl phosphates are widely employed as solvents, lubricants, plasticisers, in making paints and varnishes and in the tanning of leather. TOCP is considered an undesirable isomer in the tri-cresyl phosphates manufactured for extensive industrial uses because of its neurotoxicity.

Triorthocresyl phosphate, tri-cresyl phosphate, tri-tolyl phosphate or tri-o-methylphenyl phosphate are all synonyms for the same compound. Triorthocresyl phosphate (TOCP) is the name most commonly used. The structural formula of tri-o-cresyl phosphate is as follows:



Tri-o-cresyl Phosphate

HISTORICAL

2. Tri-o-cresyl phosphate, now an established, notorious, neurotoxic material, has been shown to be responsible for several outbreaks of flaccid paralysis in the past thirty-six years. In 1930, during the days of prohibition on the North American continent, a rather novel type of polyneuritis, disastrous in its results and obscure in origin, afflicted thousands of people throughout the United States of America. Approximately twenty thousand human beings became paralysed and many were permanently disabled. The common denominator for all such patients was the antecedent ingestion of the so-called 'Fluid Extract of Ginger' or "Jake". However, this Jake was not the true Fluid Extract of Ginger (U.S.P.) but an adulterated form in which many bland adulterants had been substituted for the true oleoresin of ginger. This drink used to be sold at many confectionaries and drug stores under the trade name of "Fluid Extract of Jamaica Ginger, U.S.P.". It became logical to conclude that the outbreak could not be a pure alcoholic multiple neuritis or due to the tincture of ginger per se, because the epidemic was spontaneous in origin and was spread over a wide area. The rationale for such conclusions was that most of the victims freely admitted that they had consumed such a beverage for at least one to five years without any harmful effect.

Smith and co-workers (1930) are credited with establishing the causal-effect relationship of "Jamaica Ginger" paralysis in vertebrates. Their splendid chemical and toxicological investigations conclusively confirmed that the suspected fluid extract of ginger was contaminated with TOCP. Subsequently it became evident that without knowledge of its toxicological properties, TOCP had been added as an alternate bland adulterant in the illegally produced drink.

After the Jamaica Ginger outbreak in the United States, similar epidemics of flaccid paralysis from TOCP intoxication have been reported from Germany (Kidd and Langworthy, 1933), Switzerland (Jordi, 1952) and Morocco (Smith and Spalding, 1959). In Germany a number of cases of polyneuritis had followed the use of "Apiol", a parsley extract illegally ingested to provoke abortion (Jordi, 1952). The clinical and pathological findings were identical with those described in Jake paralysis (Kidd and Langworthy, 1933; Jordi, 1952). A similar incident occurred in Switzerland in 1940, where a company of 80 Swiss soldiers became poisoned by the ingestion of TOCP which had been put into olive oil cans by mistake and was later used to grill cheese sandwiches (Jordi, 1952). In 1959 TOCP was once again incriminated as the cause of a mass outbreak of paralysis in Morocco (Smith and Spalding, 1959). More than two thousand Moroccans suffered from flaccid paralysis. This outbreak proved to be due to poisoning by TOCP, present in lubricating oil which was sold as "olive oil" and subsequently used for cooking purposes by the natives of Morocco.

TOXICITY IN MAN

3. Detailed accounts of both the clinical and histopathological picture are well documented (Smith and Lillie, 1931; Goodale and Humphrey, 1931; Lillie and Smith, 1932; Burley, 1932; Aring, 1942; Hunter, 1955 and Kidd and Langworthy, 1933). The following account was typical of the clinical picture in man after poisoning by tri-orthocresyl phosphate. The characteristic feature of poisoning was that the onset of ataxia was always preceded by a symptomless period averaging about 14 days but ranging from 3 to 30 days and termed the "preparalytic stage". Then the patient could feel a tingling numbness, fatigue or cramping pain in his calf muscles which lasted for 1 to 2 days. This was followed by the rapid onset of complete or partial paralysis of the extensor muscles of the toes and feet, "foot drop" or inability to walk or stand still. Approximately a week later the motor power of the muscles of the hands and forearms was also affected; the degree of involvement varied from slight weakness of the intrinsic muscles of the hand to complete "wrist drop". The degree and severity of paralysis was proportional to the amount of "Jake" ingested and the paralysis was always bilateral and symmetrical. Generally the motor power of the legs was more severely affected than that of the arms; the cranial nerves seemed hardly ever to be damaged and the cutaneous sensory loss was never marked. Ankle jerks were always absent. The course of illness was chronic but the mortalities were exceedingly rare. Patients with marked signs of damage to the spinal cord were usually left permanently disabled and those who showed improvement in the ability to use upper and lower limbs took 6 to 18 months to do so.

Laboratory examinations of blood, cerebrospinal fluid and urine were usually negative (Kidd and Langworthy, 1933). The amount of TOCP which will cause poisoning in man is relatively small. Hodge and Sterner (1943) quote Staehelin as saying that ingestion of as little as 0.15 grams of TOCP produces poisoning and 0.5 to 0.7 grams severe poisoning in man. However, Jordi (1952) estimated that as little as 0.1 gram of TOCP might have been sufficient to cause a moderate degree of paralysis in humans.

CUTANEOUS ABSORPTION IN HUMANS

4. Potential, industrial hazards from TOCP were not known until the Second World War when, in Germany, reports were published concerning the toxic effects of TOCP among factory workers. The men involved had to work with their exposed arms in a mixture of acetone and tri-cresyl phosphate (Jordi, 1952). Three other cases due to industrial use were observed in England by Hunter et al. (1944). Studies made by Hodge and Sterner (1943) with P^{32}-labelled TOCP proved that this material could readily be absorbed through the intact human skin. This information illustrates that the dangers posed by cutaneous contamination are great and people handling TOCP in industry or otherwise should be cautioned to avoid any exposure to tri-o-cresyl phosphate.

ANIMAL EXPERIMENTS

5. Once the toxic effects of TOCP were recognized in man acute toxicity experiments were undertaken in dogs, cats, rabbits, monkeys, calves and chickens, etc. Widely differing species susceptibility was noticed (Smith and Lillie, 1931). The neurotoxic effects of TOCP resembling those in humans could most easily be produced in chickens and for that reason chickens have been used extensively for neuropathological work (Cavanagh et al, 1961; Davies et al, 1960; Casida et al, 1961 and Silver, 1960). In addition, chickens have been employed for the evaluation of antidotes against TOCP (Baron and Casida, 1962; Glees, 1961 and Bleiberg and Johnson, 1965) and teratogenic effects have been demonstrated in developing chick embryos (Roger et al, 1964).

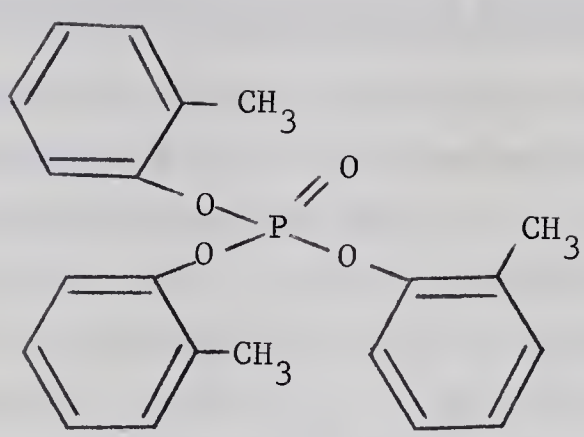
The common laboratory rodents like rabbits, guinea pigs and mice in general and the albino rats in particular are refractory to demyelination and are thus not often used for neurological studies (Hodge and Sterner, 1943).

In cats the nature of the histopathological lesions, their distribution and appearance both in the peripheral and central nervous system, resembles most closely those seen in man (Kidd and Langworthy, 1933). Quite recently Cavanagh (1964) and Cavanagh and Patangia (1965) studied the changes in the peripheral and central nervous system in cats as the result of TOCP poisoning. These workers clearly showed that the pattern of damage encountered in this species was analogous to a "dying back" process, that is, the degeneration of the nerve fibres was centripetal, both in the peripheral and the central nervous system. The conclusion drawn from the observations upon the peripheral nerves of cats was that the fibres of largest diameter and greatest length were more prone to be affected than other types of fibre. The distal neuropathy so produced affected both motor and sensory nerve fibres and occurred in the presence of apparently normal nerve cells (Cavanagh, 1964). In the cat tri-o-cresyl phosphate

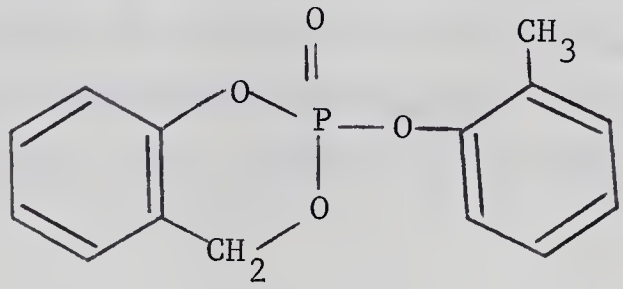
causes ataxia, preceded by a latent period of 7 to 14 days. After this time cats show an ascending, flaccid paralysis which always begins from the tail and hind legs and may increase to involve the muscles of the forelegs and trunk. The cause of death is either respiratory (bulbar) paralysis or cachexia (Kidd and Langworthy, 1933).

The minimum, effective dose in the cat is about 0.2 millilitres per kilogram, and in the dog about 1 millilitre per kilogram (Smith et al, 1930). Kidd and Langworthy (1933) report that daily subcutaneous injections of very small and, in themselves, ineffective doses of tri-o-cresyl phosphate in the cat over a period of several days are certain to give rise to flaccid paralysis of the extremities. These workers argue that TOCP is a cumulative poison and is probably eliminated very slowly from the system. Similar accumulative effects of this poison were also observed in chickens by Smith and his co-workers (1930).

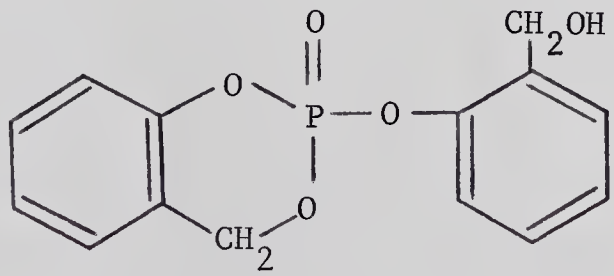
6. Aldridge (1954) and Myers and co-workers (1955) reported the biotransformation of TOCP into active anti-esterases in vivo, which could also be accomplished by liver slices in vitro (Aldridge, 1954). Similar results could be achieved by the soluble plus microsomal system of rat liver fortified with diphosphopyridine nucleotide and magnesium ions (Davison, 1955). The chemical nature of the metabolite was not elucidated until 1962, when Eto and his colleagues isolated three metabolites of TOCP from rat liver and intestine and suggested that the major pathways involved in the metabolism of TOCP were methyl hydroxylation, hydrolysis and cyclization. The three metabolites with anti-esterase activity were resolved by adsorption and partition column chromatography and they were designated in the order of their elution as metabolite 1 or M-1, metabolite 2 or M-2 and metabolite 3 or M-3. The most powerful inhibitor was M-1. This major esterase inhibiting metabolite formed in vivo has been highly purified by repeated adsorption and partition column chromatography and its biological activity determined (Casida et al., 1961). The cyclic phosphate, 2-(o-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one was also synthesized and its chromatographic properties and infrared spectrum were shown to be the same as those for M-1 (Eto et al., 1962). This product was therefore, designated as synthetic M-1 or SM-1. It was also shown to cause ataxia in hens (Baron et al., 1962) and cats (Taylor, 1965b) comparable with that produced by TOCP. The structural relationship of the parent compound (TOCP) and its two major cyclic phosphates (M-1 and M-2) is shown below:



Tri-o-cresyl phosphate (TOCP)



2-(o-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one (M-1 or SM-1)



2-(o-Hydroxymethyl-phenyl)-4H-1:3:2-benzodioxaphosphoran-2-one (M-2)

POTENTIATION OF TOXICITY IN VERTEBRATES

7. It has been demonstrated that TOCP potentiates the toxicity of malathion (0,0-dimethyl S-(1, 2-dicarbethoxyethyl) phosphorodithioate) in a few mammalian species (Murphy et al., (1959); Casida, (1961). Recently Casida et al., (1961) observed that the two major metabolites of TOCP can markedly increase malathion toxicity in mice. Thus the intraperitoneal LD_{50} for mice was 150 mg/kg. for each of anti-esterase metabolites I and II. Simultaneous injections of malathion (LD_{50} 1,500 mg/kg.) in a 10:1 ratio with the cyclic phosphates yielded LD_{50} values of about 1/45th those of either component alone. Murphy and others (1959) postulated that the potentiation depends upon the selective inhibition of esterases involved in the detoxification of malathion by TOCP metabolites. This interesting observation has lately been confirmed by Casida (1961).

TRI-O-CRESYL PHOSPHATE AND DEMYELINATION

8. It is now widely agreed that TOCP causes demyelination both in the peripheral nerves and the central nervous system (Cavanagh, 1954; Cavanagh and Patangia, 1965 and Glees, 1961). Cavanagh (1954) is of the opinion that the initial seat of damage is the axon and the myelin breakdown is secondary to axonal destruction, the condition being analogous to amyotrophic lateral sclerosis and beri beri in man. Regardless of the source of initiation of the histopathological lesions, the mechanism of demyelination after TOCP intoxication remains an enigma and a challenge for man. Several interesting biochemical hypotheses have been advanced in the past to elucidate the process of demyelination in TOCP-poisoned animals.

The Swiss neurologists, Block and Hattinger (1943) put forth a biochemical hypothesis regarding the probable way in which TOCP produced its effects and according to Glees (1961) these authors concluded that TOCP inhibited the action of cholinesterase and therefore it interfered primarily with muscle metabolism (possibly at the motor end plates) and not with the central nervous system. They found that all their animals suffered from creatinuria, which they regarded as further proof that TOCP affected muscle metabolism, but this condition could be counteracted by vitamin E, provided it was given early enough. About nine years later Earl and Thompson (1952) suggested that the nervous lesions might be associated with the anticholinesterase activity of TOCP in vivo, but soon after Davison (1953) demonstrated that some other organophosphorus compounds, which were equally effective as inhibitors of cholinesterase, did not cause demyelination or ataxia. Moreover, it was reported that tri-p-ethylphenyl phosphate (TPEP) also possessed neurotoxic properties in chickens (Bondy et al., 1960, Silver, 1960 and Cavanagh et al., 1961) despite the fact that this substance

showed very little cholinesterase activity (Aldridge and Barnes, 1961). Such findings have confirmed the belief that there is no direct relationship between the cholinesterase inhibition and the ataxia and demyelination produced by TOCP.

Susser and Stein (1959) suggested an alternative hypothesis which was rather more attractive than the previous ones. These workers argued that TOCP might interfere with myelin formation by acting as an anti-metabolite of an enzyme system concerned in the metabolism of lecithin. This postulate was based on the fact that lecithin, an important constituent of myelin, was related in chemical structure to TOCP, both compounds being esters of phosphoric acid. Moreover, Nelson and Barnum (1960) presented evidence which indicated that isoflurophate (DFP) interfered with the final stages of lecithin formation. Indeed, this hypothesis concerned with the inhibition of phospholipid synthesis and myelin sheath degeneration no longer appears to be tenable insofar as TOCP is concerned. A recent report by Taylor (1965a) indicated that TOCP did not interfere with the incorporation of C^{14}-labelled serine, choline and ethanolamine into the phospholipids of the spinal cord of cats that had developed ataxia as a result of TOCP intoxication. Ansell and Chojnacki (1965) also demonstrated that DFP did not interfere with the formation of phosphatidyl-choline in the rat brain.

Therapeutic measures such as atropinization, dosing with cholinesterase reactivators such as 2-pyridine aldoxime methiodide (2-PAM) or other oximes, hormones and vitamins have all been ineffective in the prevention of ataxia by either TOCP or its metabolite SM-1 in chickens (Baron and Casida, 1962). However, the course of the neurotoxic syndrome produced by TOCP could be slightly altered by cortisone acetate treatment in the poisoned birds (Glees, 1961, and Baron and Casida, 1962).

Although Eto et al (1962) have demonstrated the metabolism of TOCP in rats, yet this species is not susceptible to demyelination. Three metabolites of TOCP, similar in chromatographic characteristics

to those formed by rats were extracted from the feces of TOCP treated chickens by the same workers. Apparently, M-1 can be formed in both these species, one which is resistant to demyelination but capable of forming M-1 and the other highly susceptible to both TOCP and SM-1 poisoning and which shows myelin degeneration in the peripheral as well as central nervous system.

STATEMENT OF THE PROBLEM

9. The present work was undertaken to determine whether the cat metabolizes TOCP to yield M-1. TOCP produces damaging effects upon the nervous system of the cat and tends to mimic the chronic degenerative condition due to TOCP poisoning in man (Smith and Lillie, 1931; Kidd and Langworthy, 1933; Cavanagh, 1964, Cavanagh and Patangia, 1965). It has already been reported from this laboratory that SM-1 causes ataxia, demyelination and nerve fibre degeneration in cats (Taylor, 1965b). It was, therefore, considered important to demonstrate whether the cat is capable of metabolizing TOCP to form M-1 or not. If M-1 is not formed in this particular species, then it would be highly unlikely that this agent is actually responsible for the neurological lesions in cats.

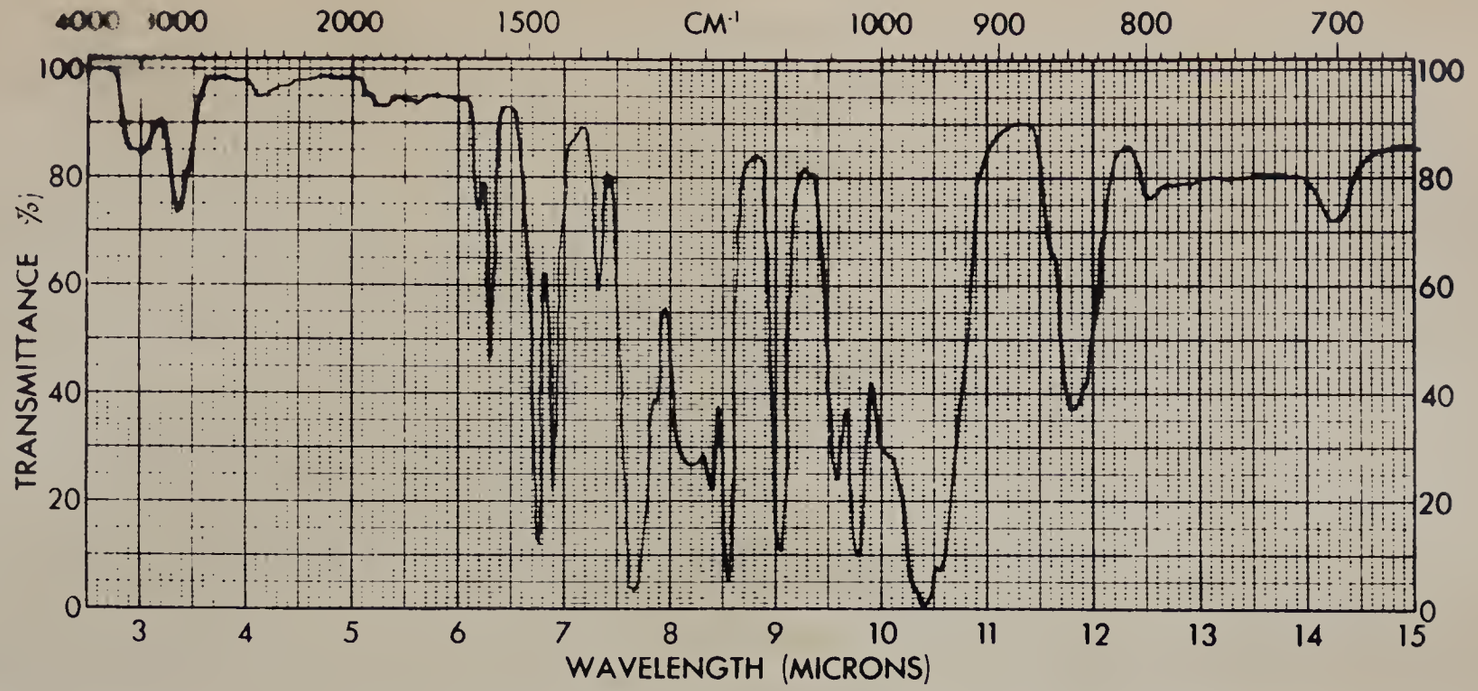
METHODS AND MATERIALS

Chemicals

1. Tri-o-cresylphosphate (practical grade) was obtained from Eastman Kodak Company, Rochester, N.Y. The practical grade TOCP was tritiated by the Wilzbach technique (1957). The labelling was done at our request by New England Nuclear Corp., Boston, Massachusetts. The radioactive material was purified to constant specific activity by adsorption column chromatography before use (Eto et al., 1962). All chemicals used for the extraction of the metabolite were procured from Fisher Scientific Company, Fair Lawn, N.J. Atropine Sulphate (U.S.P.) was obtained from the British Drug Houses (Canada) Ltd., Toronto, Canada. BBOT, 2,5-bis-[2-(5-tert-Butylbenzoxazolyl)]-Thiophene (Scintillation Grade) was obtained from Packard Instrument Company, Inc., Illinois. Tween 80, Tween 21, and Tween 60 were obtained from Atlas Powder Company, Canada, Ltd., Brantford, Canada.

Synthesis of 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one

2. SM-1, a cyclic phosphate of TOCP, was prepared according to the method of Eto, Casida and Eto (1962). O-hydroxybenzyl alcohol (3.65 gm) and o-cresylphosphoryl dichloride (6.65 gm) were dissolved in chloroform. To this mixture was added 8.40 ml of triethylamine drop by drop and the reaction mixture was constantly stirred while surrounded by an ice-bath. It may be emphasized that this is an exothermic reaction, and it is imperative that the temperature be kept below 5^{o}C in order to get a satisfactory yield. The reaction mixture was kept in the refrigerator for about 12 hours to allow the reaction to go to completion. The contents were then washed with cold water, cold 0.01N hydrochloric acid, cold 0.01N sodium bicarbonate and again with cold water. Finally the mixture was dried with 10 gm of anhydrous sodium sulphate. The crude product (4.1 gm) recovered after evaporating the chloroform under reduced pressure was purified by adsorption column chromatography to yield a light brown oil. The purified product was assayed by infrared absorption spectrophotometry by preparing a 10% solution in chloroform.


4000
3000
2000
1500
CM-1
1000
900
800
700
100
80
60
40
20
0
TRANSMITTANCE %
3
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7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

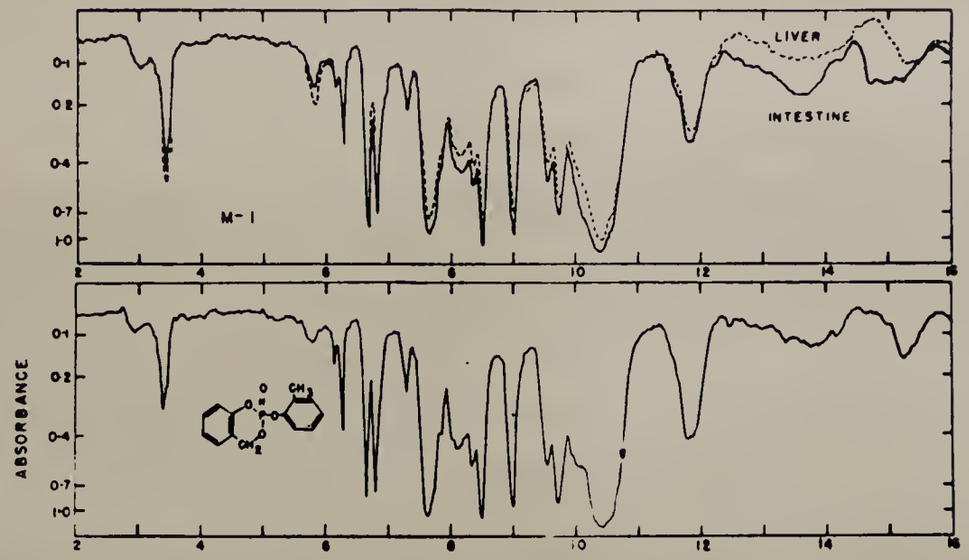
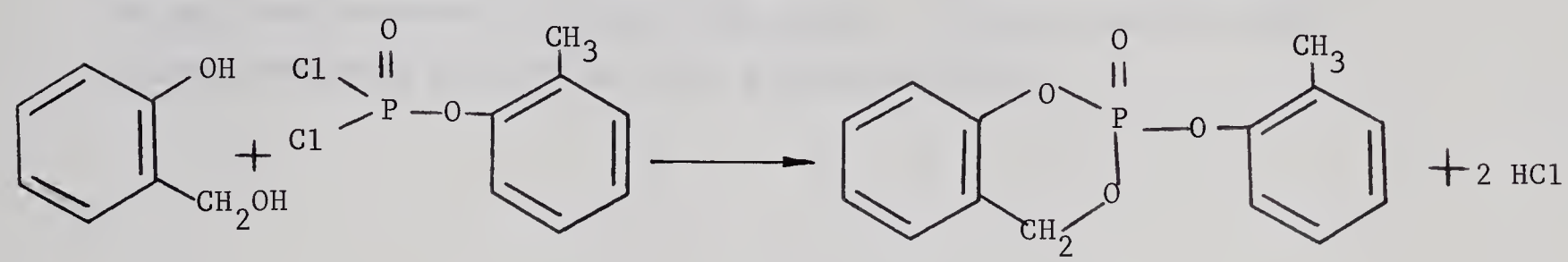

LIVER
INTESTINE
M-1
ABSORBANCE
0·1
0·2
0·4
0·7
1·0
2
4
6
8
10
12
14
16
CH3
O
O
O
O
CH2

Fig. 1. Infrared spectrogram of the TOCP metabolite, 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one in 10% Sol. of $CHCl_3$. The two smaller spectra were published by Eto, Casida and Eto (1962) and are presented here for comparison.

These spectra were almost identical to the spectrum published by Eto, Casida and Eto (1962). Copies of these spectra are shown in Fig. 1.

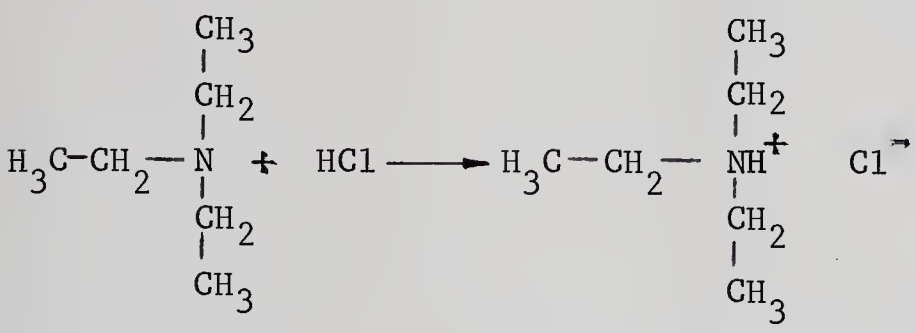
The sequence of the reactions involved in the synthesis of SM-1 is given below:



O-Hydroxybenzyl alcohol

O-Cresyl phosphoryl dichloride

SM-1



Triethylamine

Triethylammonium chloride (Triethylamine chloride)

The molar extinction coefficient for this material was 1.24 x 10^3 at 264 mμ. The phosphorus content for $C_{14}H_{13}O_4P$ was 11.22%. Found 10.8% P, after digestion in 10% perchloric acid and assayed by the method of Wahler and Wollenberger (1958). C,H and O analysis were not performed.

Chromatography

3.

(a) Adsorption Column Chromatography: The column consisted of two parts (by weight) of silicic acid and one part celite. Both the ingredients were heated before mixing for two hours at about 150°C, cooled to room temperature, slurried in hexane and packed to yield a column. The size of the column most frequently employed was 30 cm x 3 cm containing 6 gm. of the adsorbent. The benzene extracted materials were put on the column and elution accomplished with a stepwise gradient of benzene and anhydrous ether. In all cases this gradient was followed by a flush of ether to complete the elution of the compounds under investigation. Five millilitre fractions were collected.

(b) Partition Column Chromatography: Partition columns (30 cm. x 3 cm.) with 90 per cent aqueous methanol coated on celite (4 gm) as the stationary phase were used. The stationary phase was 90 per cent methanol saturated with hexane. Elution was done with hexane saturated with 90 per cent aqueous methanol.

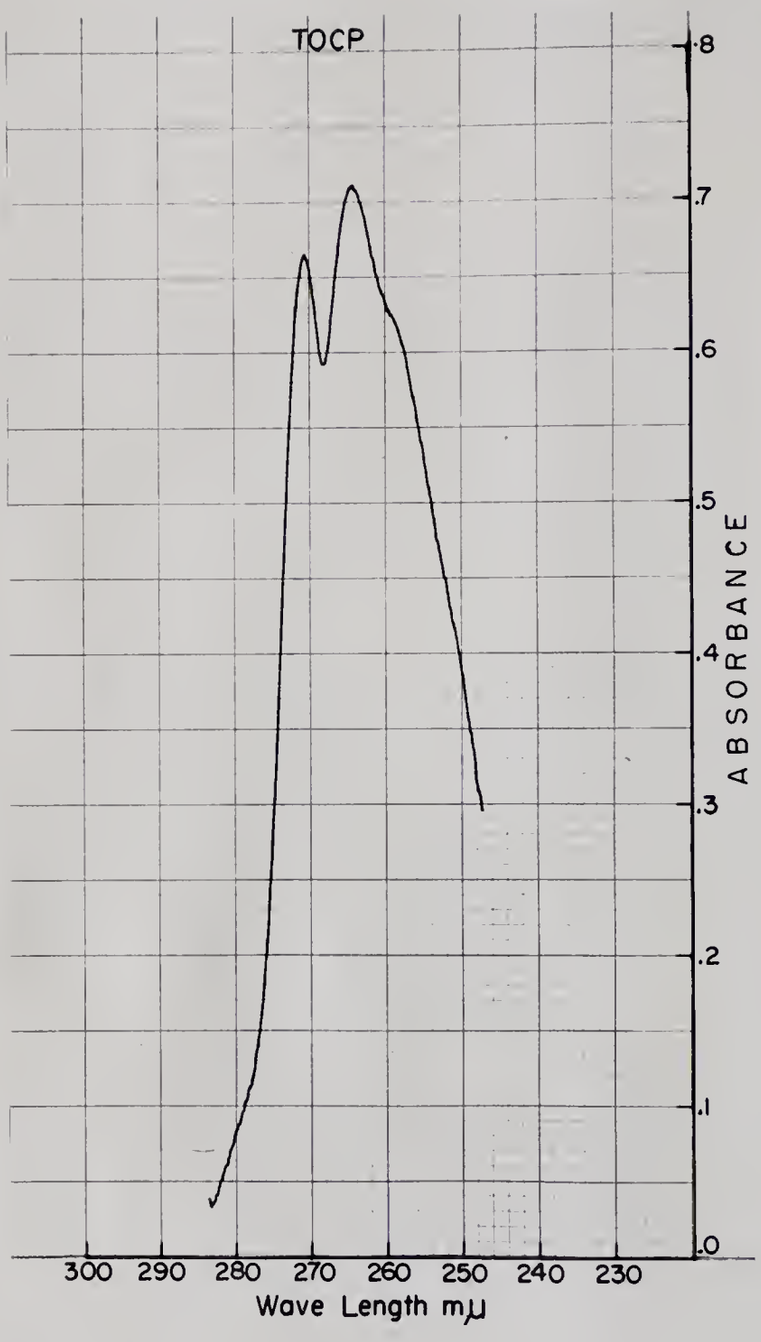

TOCP
.8
.7
.6
.5
.4
.3
.2
.1
.0
ABSORBANCE
300 290 280 270 260 250 240 230
Wave Length mμ

Fig. 2 (a). Ultraviolet spectrum of tri-o-cresyl phosphate; 155 μgm/ml in chloroform.

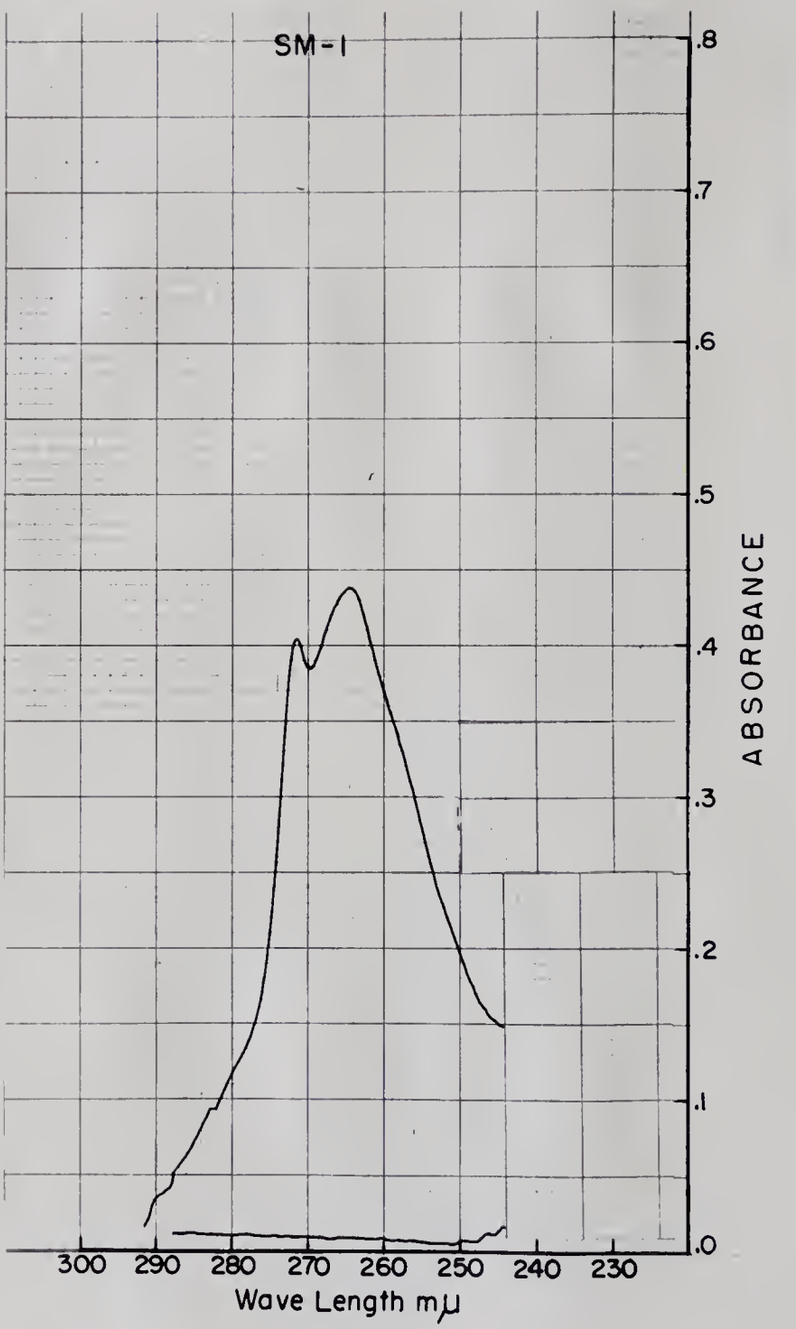

SM-1
.8
.7
.6
.5
.4
.3
.2
.1
.0
ABSORBANCE
300
290
280
270
260
250
240
230
Wave Length mμ

Fig. 2 (b). Ultraviolet spectrum of 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one (SM-1); 100 µg/ml in chloroform.

(c) Thin Layer Chromatography: A thin film (0.25 mm) of silica gel G was spread on a glass plate (20 cm x 5 cm) and air dried. The standards used for comparison were SM-1 and practical grade TOCP. Approximately 0.1 per cent solutions of standards in chloroform were used. The material under test was also dissolved in chloroform and amounts varying from 5 to 10 µl were put on the plate. The chloroform was allowed to evaporate and the samples were chromatographed without prior equilibration. The solvent front was allowed to rise about 15 cm and this took approximately 20 minutes. The solvent system consisted of isopropyl alcohol (12% V/V) in hexane.

Chromatograms were examined under ultraviolet light (short wave, UVS-12) both before and after they had been developed by spraying with 0.05 N $KMnO_4$ acidified with sulfuric acid.

Ultraviolet Spectrophotometry

4. TOCP, SM-1 and the products obtained after adsorption or partition column chromatography were subjected to spectrophotometry using a Bausch and Lomb 505 recording spectrophotometer. Fig. 2 shows the adsorption spectra of TOCP and SM-1 in chloroform between 245 to 320 mµ. When extracts were examined spectrophotometrically the presence of peaks in the region of 255-275 mµ was taken as an indication for the presence of TOCP and/or its metabolites.

Detection of Radioactivity

5. Radioactivity was determined in the liquid scintillation counter (Nuclear-Chicago Liquid Scintillation System, 725). The counting fluid consisted of a 0.4 per cent solution of BBOT in toluene. The counting efficiency in this solvent was approximately 16% as determined by the channels ratio method. Samples were counted for 10 minutes or for 1 million counts.

Animals

6. Experiments were performed on 9 cats weighing 1.5 to 4.6 kilo grams, of either sex including both young and adult animals. In most cases animals had been quarantined for two weeks, vaccinated against feline distemper and treated with appropriate anthelmintics to rid them of the internal parasites common to this species. Adult males were castrated to reduce body odour and they were kept for at least one week after castration to permit recovery from this operation. Animals were housed in individual cages and had free access to food and water. Cats were used for this study because they react with considerable regularity and exhibit the specific effects of TOCP and SM-1 on the neural tissues in a very characteristic and typical manner resembling the clinical syndrome of "ginger paralysis" observed in man.

Preparation of Emulsion

7. TOCP is a thick oily liquid which is hard to inject into animal tissues and it is practically insoluble in the usual vehicles used for parenteral administration. Although TOCP is soluble in many organic solvents like methanol and benzene these substances are themselves poisonous and TOCP was shown to increase the acute toxicity of ethanol in mice (Buttar, Tyrrell and Taylor, 1965). In view of these difficulties it was necessary to choose a vehicle which would make TOCP suitable for administration by various routes. Different combinations of TOCP were tried with the various emulsifiers such as, tween 80, tween 60 and tween 21. The following combination of TOCP and tween 80 was found quite satisfactory:

TOCP ----------	1 ml.
Tween 80 ----------	2 ml.
Distilled water ----------	17 ml.

This 5 per cent (V/V) emulsion of TOCP in 10 per cent tween 80 is quite stable and comparatively easy to inject.

Administration of TOCP

8. When administered intraperitoneally, the dose of TOCP or TOCP-H^3 was 100 mg/kg; the poison being injected as a 5 per cent V/V emulsion in 10 per cent V/V tween 80. TOCP was also injected into the shoulder muscles as a 50 per cent solution V/V in peanut oil, the dose was 0.4 ml TOCP/kg. (472 mg/kg). This dose of TOCP was found to be paralytic but sub-lethal in the atropinized cat. In order to combat the parasympathomimetic effects resulting from TOCP administration atropine sulphate was given subcutaneously at the dose of 2 to 2.5 mg. per kilogram body weight.

The intramuscular or intraperitoneal routes of administration were preferred over the oral route for the following reasons: Firstly, experience showed us that cats vomit soon after oral administration of TOCP. Secondly, evidence available in the literature indicates that the absorption of TOCP from the alimentary canal is irregular in the cat (Smith et al., 1932). In the case of acute experiments, the poison was injected intraperitoneally.

Extraction of TOCP, TOCP-H^3, SM-1 and M-1 from Cat Intestine

9. The extraction of TOCP, TOCP-H^3, SM-1 and M-1 was done essentially as described by Eto, Casida and Eto (1962).

The small intestine from a cat was weighed, chopped into small pieces and homogenized in three volumes of acetone with a Servall Omni Mixer. In some experiments either TOCP, SM-1, or both TOCP and SM-1 were added prior to homogenization. After filtration of the homogenate, acetone was evaporated under reduced pressure. To the residue was added an equal volume of saturated aqueous solution of sodium chloride and 4 times its volume of benzene. The aqueous phase was shaken twice more with 2 volumes of benzene and the combined extracts were dried with anhydrous sodium sulphate. Benzene was evaporated under reduced pressure with a rotary evaporator. The benzene soluble extractives from the tissues were then subjected to column chromatography and selected fractions from the columns were subjected to thin layer chromatography.

Recovery of SM-1 from Cat Gut Homogenate

10. In order to evaluate the losses which occurred during the extraction procedure 12 mg of SM-1, were added to the sliced small intestine from an untreated cat and homogenized with three volumes of acetone. The techniques for extraction, purification and spectrophotometric screening for the cyclic phosphate were the same as described previously.

Adsorption Chromatography of a Mixture of TOCP and SM-1

11. The purpose of this experiment was to determine whether it was possible to separate SM-1 from TOCP after the two compounds had been mixed in approximately equal proportions.

Practical grade TOCP (10.6 mg) was dissolved in 2 ml chloroform and mixed with 10.7 mg of SM-1. Chloroform was evaporated with an air jet at room temperature. Activated silicic acid (2 gm) and celite (1 gm) were slurried in 4 ml hexane and packed to yield a column. The mixture of SM-1 and TOCP was dissolved in hexane and placed on the column. Elution was done with benzene-ether mixtures as indicated in Fig. 4. Fractions of approximately 5 ml were collected and subjected to spectrophotometry in order to detect TOCP or SM-1.

Separation of SM-1 from TOCP-H^3 by Adsorption Column Chromatography

12. In this experiment a larger column of adsorbent was used together with TOCP-H^3 instead of practical grade TOCP, in order to aid in the evaluation of the resolution of these two substances by column chromatography. The greater the amount of radioactivity present in a given fraction the higher would be the concentration of TOCP-H^3 contained in it. On the other hand, material absorbing light at 265 mμ but without radioactivity associated with it, would be derived from the added SM-1.

Isotopically labelled TOCP-H^3, 9.9 mg (7.37×10^5 C/min/mg) was dissolved in 2 ml chloroform and mixed with 10.8 mg of the cyclic phosphate (SM-1). The chloroform was evaporated with an

air jet at room temperature and the mixture was dissolved in hexane and subjected to adsorption column chromatography. Activated celite (2 gm) and silicic acid (4 gm) were slurried in hexane and packed to yield a column. Void volume was about 10 ml. Elution was completed with increasing concentrations of anhydrous ether in benzene. This gradient was followed by a 30 ml flush of ether. Fractions of 5 ml were collected for assay.

Separation of SM-1 from TOCP by thin layer Chromatography

13. It was necessary to determine whether TOCP and SM-1 could be separated and identified when applied to a thin film of silica gel G as a combined material on a single spot. Practical grade TOCP (1.97 mg/ml), and SM-1 (1.02 mg/ml) were dissolved in chloroform and 10 µl of each substance were applied to a single location and at the same time 10 µl of each were also applied as separate controls. The solvent system consisted of a mixture of 12% isopropanol in hexane. The chromatograms were examined under ultraviolet light before and after they were sprayed with 0.05N $KMnO_4$ acidified with H_2SO_4 in order to locate the spots.

Identification of M-1

14. At time intervals varying from 2 hours to 6 days after the administration of either TOCP or TOCP-H^3 nine cats were killed with ether and the small intestines removed.

Acetone-extracted material from each intestine was subjected to adsorption column chromatography and the fractions thus collected were examined spectrophotometrically. Some of the fractions gave adsorption maxima in the region 250-275 mμ after the first adsorption chromatogram and therefore, they were further put to either partition or thin layer chromatography. Adsorption spectra of the fractions were obtained once again after the partition chromatography in an attempt to detect M-1.

On a thin layer chromatogram, the behavior of TOCP and M-1 under ultraviolet light and their reaction with acidified 0.05N $KMnO_4$ were used as guides for the identification of these compounds.

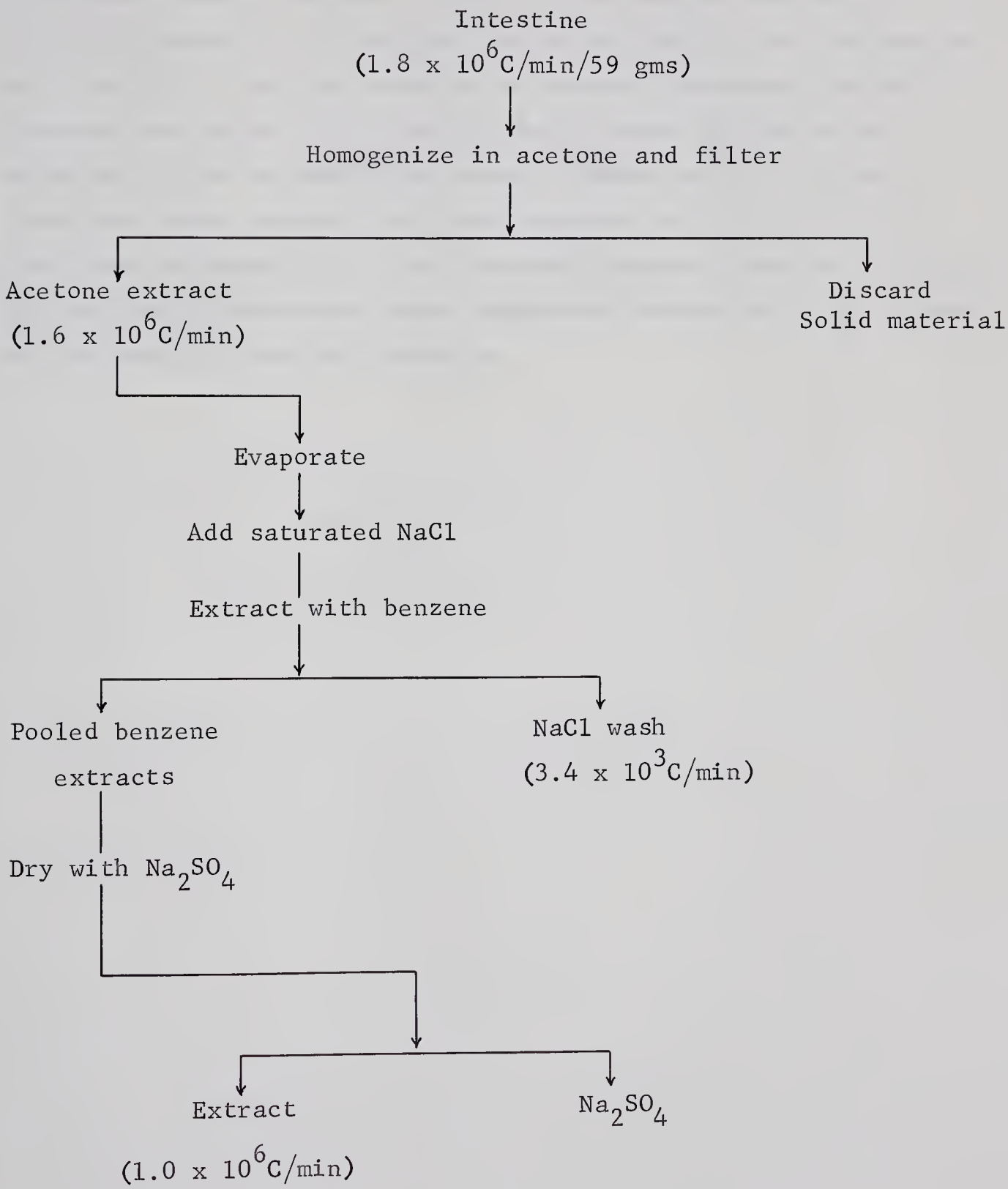
The isolation and identification of M-1 from the intestine of cat number 9 is described below to illustrate the various procedures which were employed. This cat (1.6 kg) was injected intraperitoneally with 100 mg/kg of TOCP-H^3 (1.4×10^6 d/min/mg) and killed with ether anesthesia 15 hours after the injection. Isolation of the metabolite was carried out by the method of Eto et al., (1962) as already described.

Aliquots of 0.2 ml were taken at various stages of the extraction (Fig. 3) and added to counting vials. The solvent was evaporated and 5 ml of the counting fluid was put into each vial and the number of counts per minute were determined as described previously. Fractions 11, 12 and 13 (Fig. 8) collected after adsorption chromatography were pooled because their adsorption spectra gave an indication that the amount of M-1 contained in these fractions individually was in the order of 50 μgm. Experience indicated that such a small amount of material could easily be lost on a partition chromatogram; it was therefore, decided to

subject this pooled material to thin layer chromatography. The solvent was evaporated, the residue dissolved in 0.2 ml chloroform and 10 µl of the crude extract were applied to a thin film of silica gel G together with 10 µgm of each of the reference materials, TOCP and SM-1 respectively. That portion of the chromatogram between the spot of application and the solvent front was divided into 10 equal parts. The adsorbant from each portion was carefully scraped from the glass plate and the scrapings put into separate counting vials. Five millilitres of the scintillation fluid was added into each vial and the radioactivity measured. The idea was to determine which parts of the chromatogram contained maximum radioactivity.

Figure 3

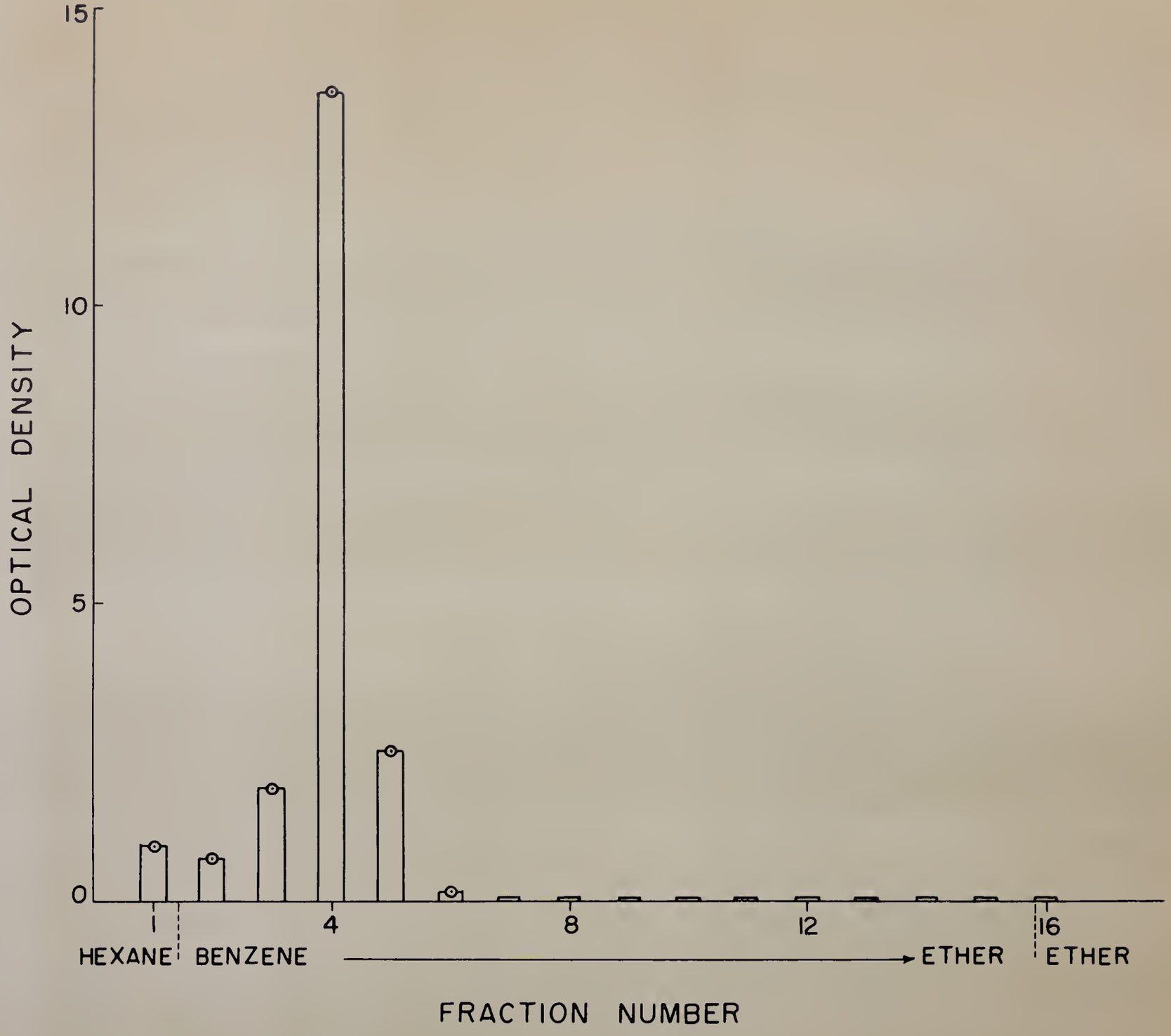
Scheme indicating the extraction of TOCP-H^3 and its tritium-labelled products



RESULTS

Early symptoms in a TOCP poisoned cat

1. It was observed that even though 2.5 mg/kg of atropine was administered subcutaneously, the cats started retching, vomiting and defecating about 20 to 30 minutes after the intraperitoneal administration of TOCP. Their pupils were widely dilated because of the atropine and no salivation and lacrimation were noticed. The time required to exhibit parasympathomimetic symptoms was about 1 to 1 and 1/2 hours after the poison was injected by the intramuscular route. These results indicated that cats could convert TOCP into esterase inhibitors which in turn were responsible for the observed parasympathomimetic effects. The following experiments were therefore, conducted in an attempt to isolate M-1 from cat intestine.


15
10
5
0
OPTICAL DENSITY
1
4
8
12
16
HEXANE
BENZENE
ETHER
ETHER
FRACTION NUMBER

The eluant composition of the fractions in Fig. 4 is given below:

Fraction No.	Eluant Composition
1	Hexane
2	Benzene
3	-do-
4	80% Benzene + 20% Ether
5	-do-
6	-do-
7	70% Benzene + 30% Ether
8	-do-
9	-do-
10	60% Benzene + 40% Ether
11	-do-
12	-do-
13	50% Benzene + 50% Ether
14	-do-
15	Ether
16	-do-

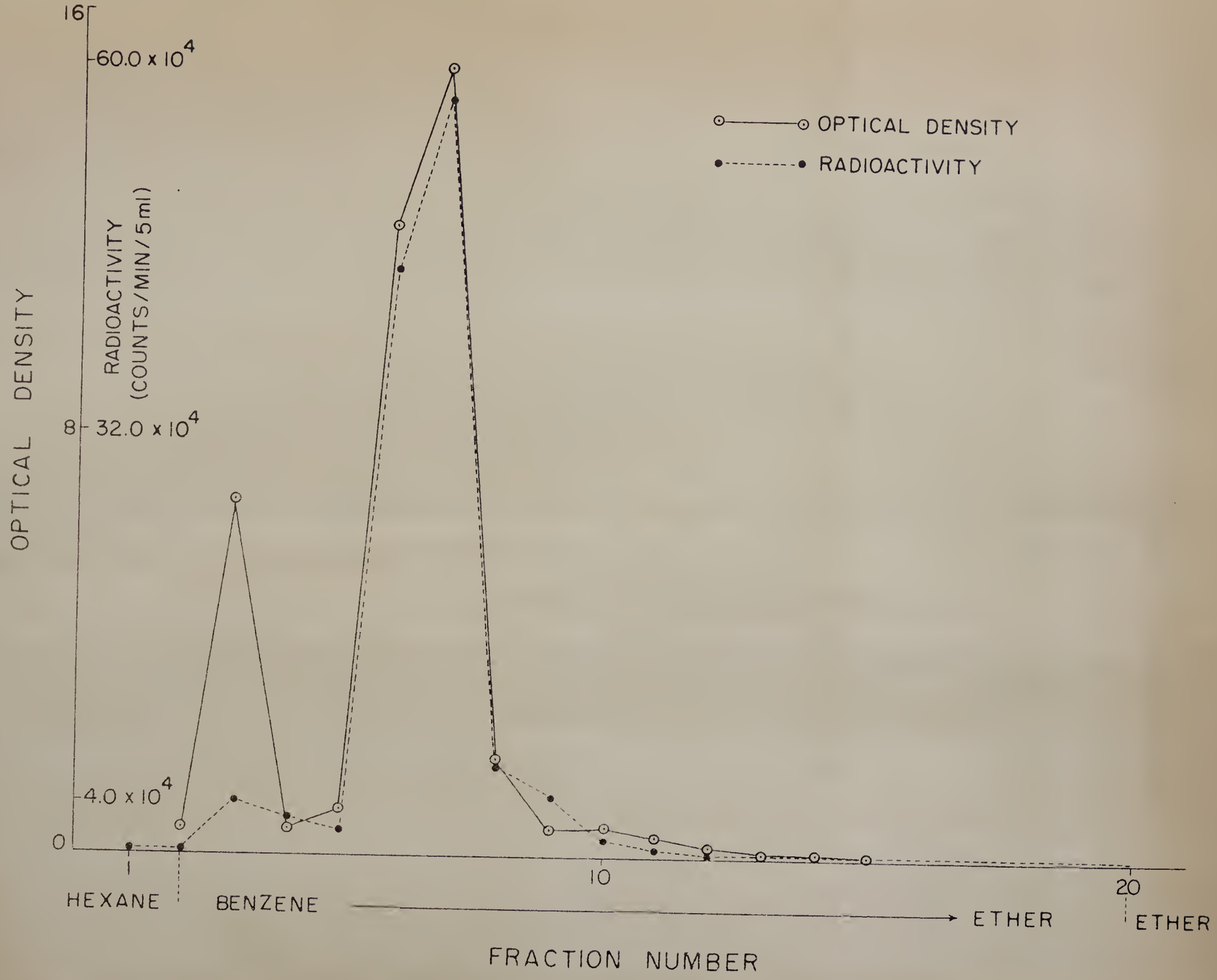
Fig. 4. Separation of SM-1 from TOCP by adsorption column chromatography on a silicic acid-celite column with a benzene to ether gradient.

Recovery of SM-1 from cat gut homogenate

2. When 12 mg of SM-1 was added to 88 gm of cat small intestine and then homogenized, extracted, and subjected to both adsorption and partition column chromatography only 4 mg was recovered. Thus this in vitro experiment resulted in 33 per cent recovery of the synthetic metabolite from the cat intestine and showed that the loss of this material during extraction and chromatography was quite significant.

Adsorption Column Chromatography of a mixture of TOCP and SM-1

3. The results of an attempt to resolve TOCP from SM-1 by adsorption column chromatography are shown in Fig. 4. The optical densities are plotted against the fraction numbers. An impurity was eluted with benzene in fractions 1 to 3. Fraction 1 had an absorption maximum at 253 mμ while fractions 2 and 3 showed a single peak at 258 mμ. Fraction 4 showed peaks at 263 and 269 mμ. The absorption spectrum of fractions 5 and 6 had peaks at 265 and 270 mμ. Only the tubes with two peaks at 265 and 270 were considered truely representative of SM-1. Fraction 4 probably contains a mixture of TOCP and SM-1.


16
60.0 x 10^4
8
32.0 x 10^4
4.0 x 10^4
0
OPTICAL DENSITY
RADIOACTIVITY (COUNTS/MIN/5ml)
OPTICAL DENSITY
RADIOACTIVITY
10
20
HEXANE
BENZENE
ETHER
ETHER
FRACTION NUMBER

The eluant composition of the fractions in Fig. 5 is given below:

Fraction No.	Eluant Composition
1	Hexane
2	-do-
3	Benzene
4	-do-
5	-do-
6	-do-
7	80% Benzene + 20% Ether
8	-do-
9	-do-
10	-do-
11	70% Benzene + 30% Ether
12	-do-
13	-do-
14	-do-
15	60% Benzene + 40% Ether
16	-do-
17	-do-
18	50% Benzene + 50% Ether
19	-do-
20	Ether
21	-do-

Fig. 5. Adsorption column chromatography of a SM-1 and TOCP-H[3] mixture on a silicic acid-celite column with a benzene to ether gradient.

Adsorption Column Chromatography of TOCP-H^3 and SM-1

4. Figure 5 shows the optical density and radioactivity present in each fraction collected after adsorption column chromatography of a mixture of TOCP-H^3 and SM-1. The substance eluted with benzene in fractions 2 and 3 contains small amounts of radioactivity. This material had a single absorption maxima at 255 mμ. Fraction number 6 showed peaks at 260 and 270 mμ. The maximum amount of radioactivity was associated with fraction number 7 (5.7 x 10^5 C/minute/5 ml) which gave absorption peaks at 265 and 270 mμ. Once again, a single peak at 265 mμ was noticed in each of the fractions 8, 9 and 10. There was a small amount of radioactivity associated with each of these fractions as can be seen from Figure 5. Fractions number 11, 12, and 13 showed absorption occuring at 265 and 270 mμ but the amount of material was very small i.e., the radioactivity recorded in them was in the order of 4.5 x 10^3 C/min/5 ml.

In order to estimate the amount of material eluted from the column, fractions 6, 7, 8, 9 and 10, which contained the greatest amounts of the material were pooled. The chloroform was evaporated with an air jet and the resultant material thus recovered was 13.2 mg. It constituted 64 per cent of the original TOCP-H^3 and SM-1 mixture (20.7 mg) put on the column.

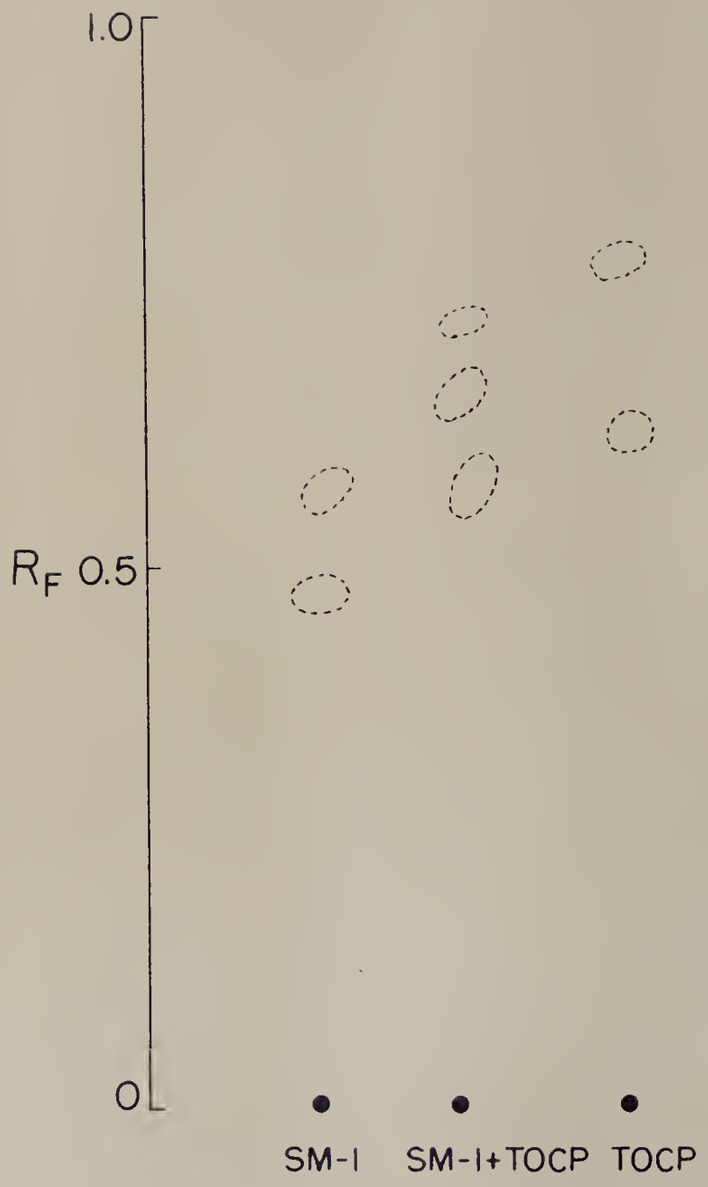

1.0
R_F 0.5
0
SM-1
SM-1+TOCP
TOCP

Fig. 6. Thin layer chromatography of TOCP, SM-1 and a mixture of TOCP and SM-1 on silica gel G.

Separation of TOCP from SM-1 by thin layer chromatography

5. Although SM-1 occasionally gave a single spot, it usually appeared as two discrete spots (Rf 0.48 and 0.59) on chromatograms (see Fig. 6). Both these SM-1 spots showed similar qualities under the ultraviolet light, that is, both showed blue fluorescence and both developed a yellowish colouration when sprayed with acidified potassium permanganate solution.

TOCP also gave two spots (Rf 0.63 and 0.78) which gave a yellow fluorescence under the ultraviolet lamp but did not develop when the chromatogram was sprayed with 0.05 N $KMnO_4$.

When 10 µgm of SM-1 and 20 µgm of TOCP were put on the same spot, there appeared three discrete spots on the chromatogram (Rf-values 0.57, 0.65 and 0.73) and all of them were visible under the ultraviolet light. After development with $KMnO_4$, only one of these spots appeared as a pale spot having Rf-value 0.57. It was apparently a SM-1 spot because TOCP did not develop with the spray reagent being used. These results can be seen in Fig. 6.

Only the spots at Rf 0.59 (SM-1), 0.57 and 0.65 (SM-1 + TOCP), and 0.63 (TOCP) were the major spots. The other spots represented minor components which were present in concentrations that were just detectable and this may account for the absence of one spot from the mixture of SM-1 and TOCP.

Identification of M-1

6. The results of attempts to recover M-1 or SM-1 from the intestines of 8 cats, which either died or were killed at varying times after the administration of TOCP or TOCP-H^3 are presented in Table I. The result of an experiment on the recovery of SM-1 from cat intestine is also included in this table. Cat number 1 was given 0.4 ml TOCP/kg. intramuscularly and it died three days after the injection. Cat number 2, was injected with an equivalent amount of poison died six days after the treatment. Acetone extracted material from the intestines of these two animals was subjected to adsorption column chromatography and the different fractions were examined spectrophotometrically. No M-1 was detected in either of these cases.

Table I

Attempts to recover M-1 from cat intestine

Cat No.	Weight of Cat (kg)	TOCP (mg/kg)	Route of Administration	Time Interval from Injection (hrs)	Result
1	4	472	I.M.	72 (died)	Negative
2	4.6	472	I.M.	144 (died)	Negative
3	4.5	None	12 mg SM-1 homogenized with intestine	---	33% of SM-1 recovered.
4	1.5	472	I.M.	24	M-1 like material was detected with T.L.C.
5	2.7	472**	I.M.	12	Negative
6	2.4	400**	I.M.	36	M-1 like material detected with T.L.C.
7	2.7	100	I.P.	40 (died)	Negative
8	1.8	100**	I.P.	2	Negative
9	1.6	100**	I.P.	15	M-1 like material detected with T.L.C.

Note: I.M. = Intramuscular I.P. = Intraperitoneal

T.L.C.= Thin layer chromatography

** TOCP-H^3

Results of three experiments where TOCP-H^3 or TOCP was injected into cats and a M-1 like material was isolated are set out in Table II.

Table II

Thin layer chromatographic evidence for M-1 as a product of tri-o-cresyl phosphate

Cat No.	Weight of cat (kg.)	TOCP (mg/kg.)	Route of Administration	Time Interval from injection (hrs)	Rf-values
4	1.5	472	I.M.	24	0.58 (0.57)*
6	2.4	400**	I.M.	36	0.57 (0.58)
9	1.6	100**	I.P.	15	0.56 (0.59)

Note: I.M. = Intramuscular I.P. = Intraperitoneal

*Rf-values of the reference (SM-1) are given in parenthesis for purposes of comparison.

** TOCP-H^3

The Rf-value of the reference SM-1 did not agree perfectly with the material isolated however, it appeared to be similar to SM-1 when its location, fluorescence under ultraviolet light, and reaction with $KMnO_4$ spray reagent were all considered together.

Four to five additional spots other than the TOCP and M-1 spots appeared on chromatograms of the fractionated material. A couple of these additional spots were pink, while others were light brown in colour. An hour after spraying with 0.05N $KMnO_4$, the colour of these additional spots increased in intensity and turned dark brown. The reaction of these additional spots with potassium permanganate was quite different and hence could not be confused with either of TOCP or M-1. The M-1 spot acquired a light yellow colour with $KMnO_4$, while TOCP did not react with this spray reagent.

The extraction of tritium-labelled products following intraperitoneal administration of TOCP-H^3

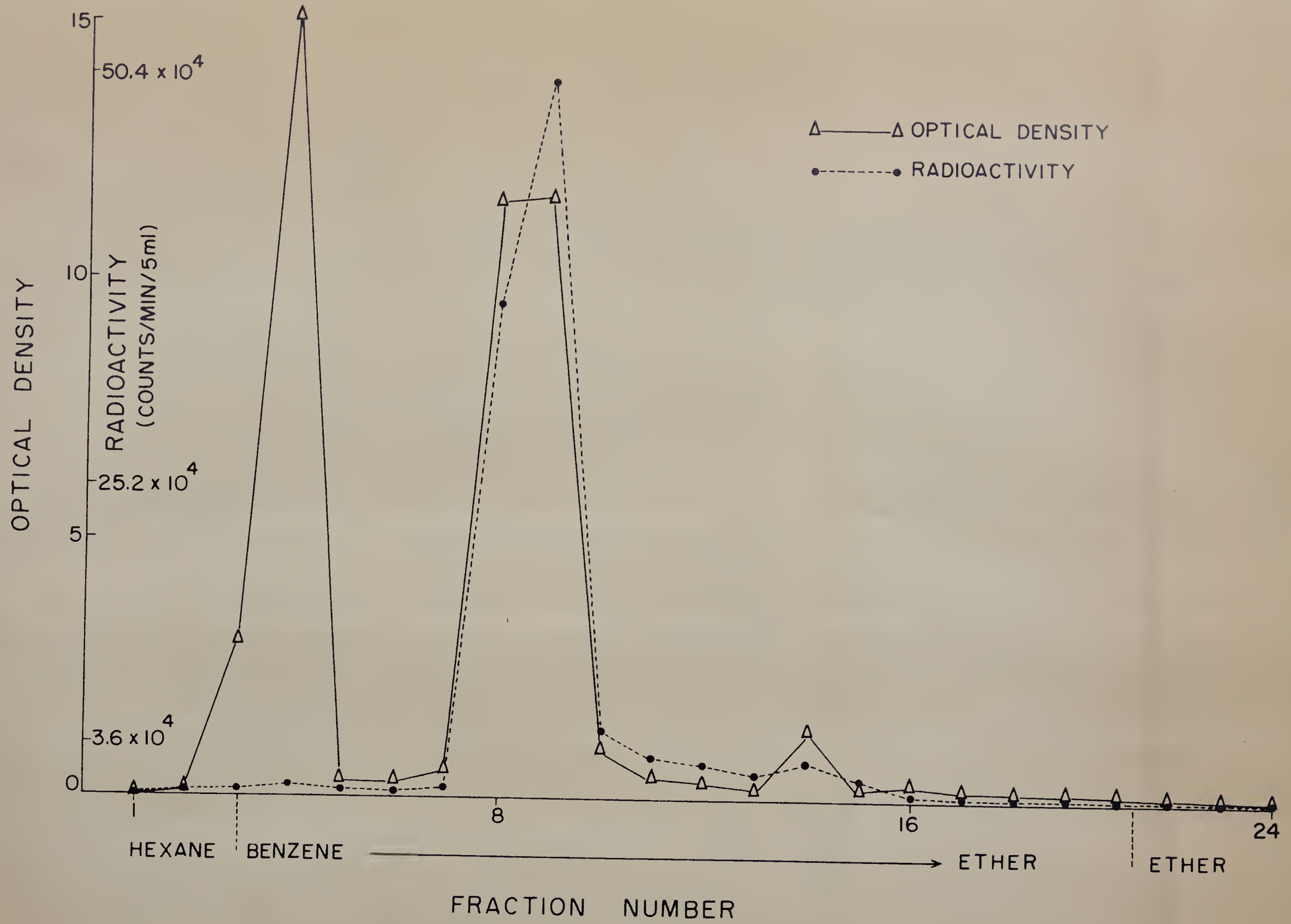
7. Since previous experiments had shown that the loss of SM-1 when mixed with cat gut homogenates and chromatographed was quite marked; efforts were made to determine the loss of tritium-labelled material following intraperitoneal administration of TOCP-H^3 and during the course of the isolation of M-1-H^3. Table II shows the losses encountered at different stages of extraction and purification. Approximately 11% of the total activity in the intestine was lost during acetone extraction and about 33% was lost through drying with anhydrous sodium sulfate.

Only traces of radioactivity (0.2%) were lost in the residue which had been washed with saturated sodium chloride and later extracted with benzene. The losses of radioactivity on the adsorption column were negligible since all of the radioactive products put on the column were eluted from the latter. Thus the final recovery of tritium from the intestine was about 56%.

Table III

Evaluation of the loss of extractable material from the intestine of a cat injected with TOCP-H^3 (100 mg/kg)

		Cumulative Loss
Radioactivity in 59 gm of intestine	1.8×10^6 C/min.	--
Radioactivity in 220 ml acetone extract	1.6×10^6 C/min.	11%
Radioactivity in 181 ml benzene extract	1.0×10^6 C/min.	44%
Radioactivity eluted from the column	1.0×10^6 C/min.	44%


15
50.4 x 10^4
10
RADIOACTIVITY
(COUNTS/MIN/5ml)
25.2 x 10^4
5
OPTICAL DENSITY
3.6 x 10^4
0
OPTICAL DENSITY
RADIOACTIVITY
1
8
16
24
HEXANE
BENZENE
ETHER
ETHER
FRACTION NUMBER

The eluant composition of the fractions in Fig. 7 is given below:

Fraction No.	Eluant Composition
1	Hexane
2	-do-
3	-do-
4	Benzene
5	-do-
6	-do-
7	-do-
8	80% Benzene + 20% Ether
9	-do-
10	-do-
11	-do-
12	70% Benzene + 30% Ether
13	-do-
14	-do-
15	60% Benzene + 40% Ether
16	-do-
17	-do-
18	50% Benzene + 50% Ether
19	-do-
20	-do-
21	Ether
22	-do-
23	-do-
24	-do-

Measurements were made at the wave length which gave the maximum optical density, provided it was within the region of 255-275 mμ.

Fig. 7. Optical density and radioactivity in different fractions after adsorption column chromatography of an extract from the intestine of a TOCP-H^3 treated cat.

Fig. 7 illustrates the results of the absorption spectra of different fractions determined after adsorption column chromatography of material isolated from the intestine. Fraction number 8 and 9 showed peaks at 259 and 269 mμ while fractions 11, 12 and 13 gave peaks which were at 263 and 270 mμ. Higher concentrations of material were found in fractions 8 and 9 as compared with fractions 11, 12 and 13. An unidentified substance showing a single peak at 253 mμ was eluted with benzene in fractions number 3 and 4.

It is evident from Fig. 7 that the maximum radioactivity was associated with fractions number 8 (3.4×10^5 C/min/5 ml) and 9 (5.0×10^5 C/min/5 ml). On the other hand, the amounts of radioactivity present in fractions 11, 12 and 13 were comparatively low, ranging from 1.6×10^4 to 2.9×10^4 C/min/5 ml. It is important to note that only traces of radioactivity were detected in fractions 3 and 4 which had a high optical density. Therefore, this impurity is of biological origin and unrelated to the TOCP-H^3 which was administered.

The thin layer chromatographic results are presented in Fig. 8 and plate 1. A single SM-1 spot (Rf 0.59) with blue fluorescence, and two TOCP spots (Rf-values 0.64 and 0.79) having a light yellow fluorescence were noticed on a chromatogram which was exposed to ultraviolet light. The reference TOCP spots with Rf-0.64 was larger than at the spot at Rf 0.79. From the test material a spot of Rf-value 0.56 gave a blue fluorescence and another Rf-value 0.64, showed a light yellow fluorescence under the ultraviolet light. The boundaries of all these spots were marked for further reference. A light yellow colour was seen on the developed chromatogram in one of the test spots, Rf 0.56; and the reference SM-1 spot, Rf 0.59. The other test spot, Rf 0.64, and the two reference TOCP spots did not develop with this spray reagent.

The fluorescence characteristics of all the above described spots, including the test and reference, did not alter after treatment with $KMnO_4$. Furthermore, it may be noticed from Fig. 8 that although the test spots marked as (a) and (b) are discrete spots, their Rf-values are quite close to each other,

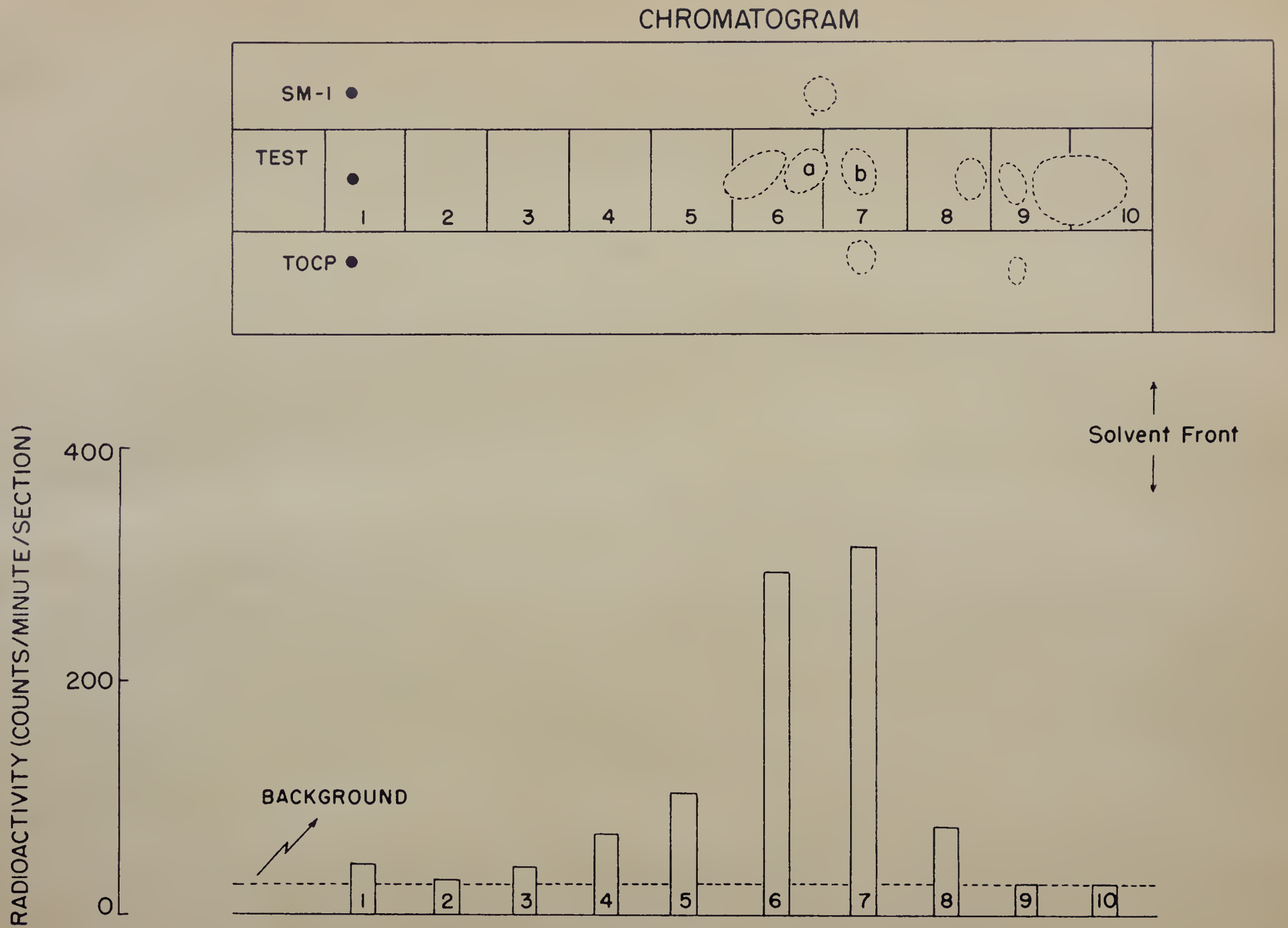

CHROMATOGRAM
SM-1
TEST
TOCP
1
2
3
4
5
6
7
8
9
10
a
b
Solvent Front
RADIOACTIVITY (COUNTS/MINUTE/SECTION)
400
200
0
BACKGROUND

Fig. 8. (TOP) Thin layer chromatogram of an extract from the intestine of TOCP-H^3 treated cat; (BOTTOM) radioactivity associated with each portion of the chromatogram.

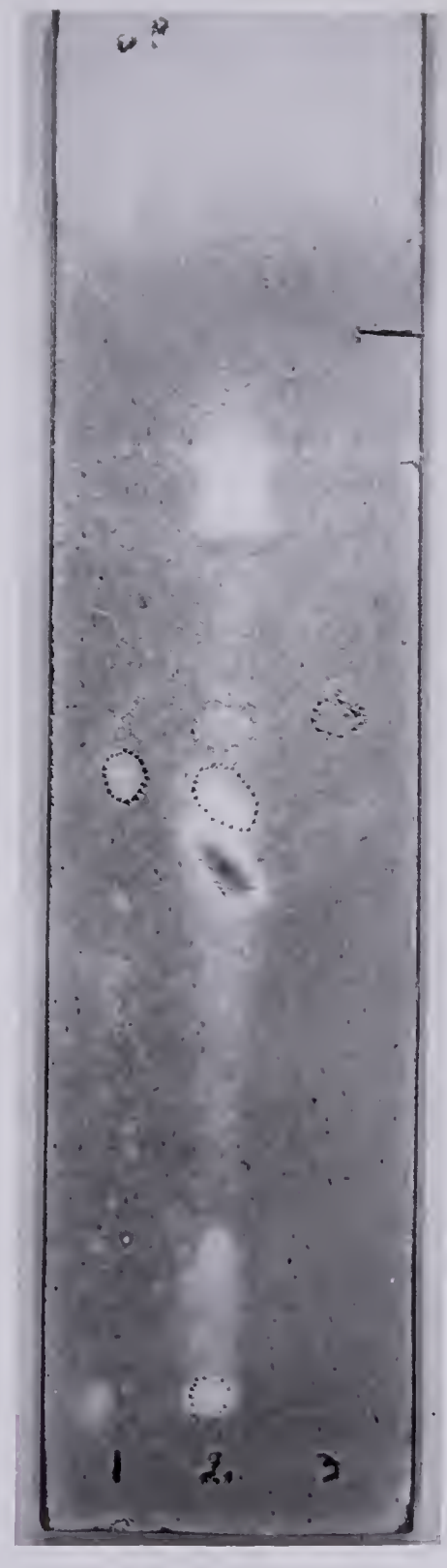


Plate 1. Photograph of a thin layer chromatogram showing M-1 separated from TOCP-H^3

1. SM-1 (1.03 mg/ml)
2. Test
3. TOCP (1.01 mg/ml)

viz., 0.56 and 0.64 respectively. It may also be seen from Fig. 8 that the test spot (a), Rf 0.56; corresponds roughly with the reference SM-1 spot (Rf 0.59) whereas, spot (b) of the test, Rf 0.64; matches the standard TOCP spot (Rf 0.64). It may be emphasized that the characteristics of these two pairs of spots under the ultraviolet light and with $KMnO_4$ were the same as described previously for SM-1 and TOCP and this assisted in their identification. Furthermore, the results presented graphically in Fig. 8 indicate that the maximum amounts of radioactivity were associated with the portions marked 6 and 7 on the chromatogram. These portions correspond to the segments of the chromatogram containing the spots with Rf-values 0.56 and 0.64 as described above.

Discussion

It is impractical to administer TOCP intravenously because it is a viscous oil. Its absorption, when administered by the intramuscular route, is slow and this is a disadvantage in isolation of metabolites insofar as tissue concentrations remain low for a considerable time after the injection. Furthermore, it was observed that the injection of TOCP either alone or as a 50% solution in peanut oil led to the formation of sterile abscesses in the forelimbs of cats. If these sterile abscesses drain some TOCP is lost and this raises an uncertainty as to the effective dose. The disadvantage of the oral route is that cats vomit soon after administration of the TOCP. In this regard, the emulsion of TOCP in tween 80 shows great promise, since it can be used both for intravenous and intraperitoneal injections.

Tween 80 (polysorbate 80) is a mixture of polyoxyethylene oleic acid esters of sorbitol anhydrides. Tweens are potent emulsifying agents and are widely used in pharmacy, food and cosmetics (White, 1964). Polysorbate 80 has been used in humans to improve the absorption of fats and fat soluble vitamins in conditions like steatorrhea (Grollman, 1954).

The acute parasympathomimetic symptoms following TOCP administration provided the first indication that TOCP was metabolized to an antiesterase. Large doses of atropine were ineffective in preventing all of the toxic symptoms of TOCP poisoning, for example, vomiting still persisted and the animals developed paralysis.

In preliminary trials no M-1 could be isolated from cat intestine. It was thought that either the metabolite was present in amounts too small to be isolated or that the losses incurred during the process of isolation and purification of the metabolite were so great that no material could be recovered. Recovery of SM-1 from the cat intestine homogenate indicated that losses of SM-1 added prior to extraction and purification, were quite marked.

It was pointed out by Eto et al., (1962) that the cyclic phosphate was very sensitive to hydrolysis. Indeed, these workers established that when M-1 was allowed to stand at room temperature in either acidic or alkaline solutions, its antiesterase activity diminished rapidly.

It was not possible to resolve TOCP-H^3 from SM-1 by adsorption column chromatography. Eto, Casida and Eto (1962) claimed that they successfully eluted three major metabolites of TOCP following adsorption column chromatography of acetone extracts of rat intestine and liver. They designated these fractions in the order of their elution, as M-1, M-2 and M-3 respectively. Indeed, Eto et al., (1962) published a composite diagram based on cholinesterase inhibition by M-1, M-2 and M-3 isolated from the rat intestine. TOCP was superimposed on this diagram and these workers indicated that because TOCP is less polar than its metabolites, it should be eluted just before M-1. However, no evidence was presented by these authors as to whether or not TOCP could be readily separated from M-1. Unfortunately, this diagram was misleading because it gave the impression that TOCP and its metabolites could be easily separated from each other. The slight shift in absorption maxima between fractions 6 and later fractions 7-13 (Fig. 5) indicate that there is a tendency for TOCP-H^3 to preceed SM-1 off the column but that they do not separate readily. In other words, it may be said that there was an incomplete separation of TOCP-H^3 and SM-1 from their mixture by adsorption chromatography. As a matter of interest, Eto et al., (1962) state that 24.5 gm. of material resulting from the first chromatographic purification eventually yielded 26.6 mg. of M-1 following six chromatographic purifications. This would indicate that the ultimate recovery of M-1 was of a very low magnitude following repetitive adsorption and partition column chromatography.

In view of the facts outlined above a better method was needed in order to obtain a separation of M-1 from TOCP. Thin layer chromatography on silica gel G was used to effect this separation.

Moeschlin (1965) is of the opinion that TOCP can easily be detected in remains of food and oil by its intense blue fluorescence in ultraviolet light. We have not been able to confirm these observations exactly as he described them. When a drop of practical grade TOCP was applied to a thin layer chromatogram and exposed to ultraviolet light, it had a dual fluorescence, seen to be light yellow in the center and light blue at the periphery, whereas, SM-1 gives an intense blue fluorescence under ultraviolet light. This differential fluorescence has been used to assist in the identification of M-1.

The consistant appearance of two spots on chromatograms of practical grade TOCP would indicate that this substance was not chemically pure. The reason for SM-1 appearing as two separate spots at certain times is not very well understood. It may be that the compound decomposes when in solution, or possibly water present in the solvent used for chromatography effects its decomposition.

When TOCP and SM-1 were put at the same spot, three discrete fluorescent areas were seen under the ultraviolet light. Most probably, two of these spots with Rf 0.65 and 0.73 were representatives of TOCP and the third spot with Rf 0.57 represented SM-1, because under ultraviolet light the spot at Rf 0.57 had blue fluorescence and this was the only spot which appeared to acquire a yellow colour on the developed chromatogram. On the other hand, spots at Rf 0.65 and Rf 0.73 gave light yellow fluorescence and they did not appear as coloured spots when the chromatogram was developed. These findings, therefore, suggest that it is possible to resolve a mixture of SM-1 and TOCP by thin layer chromatography. With this technique, it was possible to identify a material like M-1 from three separate cats treated with either labelled or unlabelled TOCP and killed at varying intervals of time after the injection. It is felt that the spots having Rf-values in the range of 0.56 to 0.58 belong to a M-1 like material and the spots

with Rf-values of 0.64 to 0.73 represent TOCP. It may, however, be worthwhile to mention that with the present solvent system, the spots of TOCP, SM-1 or M-1 could not be widely separated as shown by their respective Rf values (see Figs. 6 and 8). Therefore, a solvent system which will give a better resolution between these two compounds is still being sought.

The optimum time for a M-1 like material to be present in detectable amounts in cat intestine following intramuscular administration of TOCP lies between 24 and 36 hours, while after an intraperitoneal injection the optimal time appears to be about 15 hours. Eto et al., (1962) found that the antiesterase activity of an acetone-soluble fraction of intestine was maximal two hours after oral administration of TOCP (1 ml/kg) to rats. In other words, two hours was the optimum time for the metabolite to be present in maximum concentration in rat intestine.

Since a M-1 like material was isolated from the intestines of cats 15, 24 and 36 hours after TOCP administration, but not earlier or later; this indicates that the rate of metabolism of TOCP in the cat may be slower than that of the rat. Two hours time may not be sufficient for M-1 to be present in cat intestine in amounts detectable by our techniques. A large part of the radioactivity was present as TOCP which had not yet undergone metabolism to give rise to M-1.

Myers et al., (1955) injected TOCP intraperitoneally into rats and rabbits and they arrived at the conclusion that approximately 80% of the total esterase inhibitor was located in the intestinal tract. According to them, TOCP was converted into an active inhibitor in the liver, excreted in the bile and reabsorbed in the intestine where appreciable amounts of the inhibitor could still be recovered 7 to 10 days after TOCP was administered. However, no metabolites of TOCP were isolated from the intestinal tracts of cats three days and six days after the

TOCP injection. This discrepancy may be due to the species difference or owing to the different methods used for extraction of the metabolite.

The evidence gathered from various sources, that is, determination of adsorption spectra, thin layer chromatography, and examination under ultraviolet light, to identify M-1, indicates that the cat is capable of metabolizing TOCP to a substance similar to M-1. It has already been demonstrated that SM-1 is neurotoxic in cats (Taylor 1965b) and that this toxicity resembles that seen in TOCP poisoning. It is therefore, possible that the neurotoxicity of TOCP in cats may result from a metabolite or metabolites analogous to M-1.

Conclusions

1. The use of an emulsion of TOCP in tween 80 provides a convenient preparation for parenteral administration.

2. Considerable losses of SM-1 and TOCP occur during the extraction procedures employed.

3. TOCP cannot readily be separated from SM-1 by adsorption column chromatography alone, but when this was supplemented with thin layer chromatrography on silica G using 12% isopropanol in hexane as solvent, these two substances were resolved.

4. A substance similar to SM-1 in chromatographic behaviour, fluorescence under ultraviolet light, and reaction to 0.05N $KMnO_4$ was isolated from cat intestine.

5. The isolation of the metabolite of TOCP together with the appearance of signs resembling parasympathetic stimulation lead us to conclude that the cat can metabolize TOCP to a substance similar to the M-1 isolated from rat intestine by Eto, Casida and Eto (1962).

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